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ORGANIZATION AND EXPRESSION OF PLASMODIAL GENES REQUIRED FOR ERYTHROCYTE INVASION

ANNUAL REPORT

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The invasion of erythrocytes and sequestration of infected cells in the microvasculature enables P. falciparum to minimize its interaction with the host, resulting, however in the significant morbidity and mortality of falciparum malaria. The molecular basis for parasite sequestration involves a complex macrostructure elicited on the infected erythrocyte surface called the knob. The interaction of parasite and host proteins to form this structure and the genetic regulation of this structure has been investigated through the structural and functional analysis of knob associated proteins. The knob-associated histidine rich protein (KAHRP), together with parasite and host derived proteins interact to mediate cytoadherence. Characterization of the protein interactions which result in endothelial cell binding and the identification of parasite proteins involved in this phenotype have been studied through their interaction with the KAHRP. Overexpression of the cloned KAHRP gene in bacteria provided an abundant source of this protein for the preparation of monospecific antisera and for the mapping of the interactions between this protein and other host and parasite derived molecules

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which result in cytoskeletal deformation and cytoadherence. Characterization of knobless (K^-) mutants further defines the structure, function and regulation of the knob. The molecular basis for the telomeric rearrangement which result in the K^- phenotype has been under investigation through the analysis of the parent and recombinant chromosomes. The contribution of telomere structure and stability to chromosomal rearrangements has been investigated through the analysis of the factors which regulate the dynamics of telomere length.

SUMMARY

The goal of the previous year's work was to define the structure and function of genes implicated in P. falciparum invasion of erythrocytes and the sequestration of those infected erythrocytes in the microvasculature. The gene for the glycophorin binding protein of 130,000 dalton was cloned, the complete nucleotide sequence of the cDNA and gene defined and the expression of that gene in E. coli was achieved, generating the substrates for biochemical studies. Previous studies by Perkins defined this protein on the merozoite surface as involved in erythrocyte invasion. Antibodies against the native protein inhibited the re-invasion of erythrocytes by merozoites in vitro. The primary sequence of this protein was predicted to encode 11 copies of a 50 amino acid repeat sequence, preceeded by a 225 amino acid amino terminal charged domain. While the role of tandem repeat sequences in plasmodial genes is still enigmatic, a correlation between structure and function for this gene was possible. Through the overexpression and purification of recombinant protein expressing between 3-11 repeats, it was found that the strength of binding of the protein to its ligand, glycophorin, was a function of repeat number. Thus, maximal binding to glycophorin was achieved only when 11 copies of this repeat was expressed. The repeats are well conserved within the molecule as well as for different strains of P. falciparum, suggesting that a critical function for the repeat sequence has been selected. Antibodies raised against the recombinant protein (monoclonal and polyclonal) inhibited the invasion of erythrocytes by merozoites in vitro. Current studies are underway to assess the significance of this molecule in mediating invasion in vivo, utilizing the Aotus monkey model.

Studies on sequestration focussed on the isolation and characterization of the gene for the knob associated histidine rich protein. Through the use of a genomic clone isolated and characterized for an evolutionarily conserved protein in an avian parasite, the histidine-rich protein of P. lophurae, cDNA clones were isolated from a P. falciparum library constructed to mRNA isolated from the knob-expressing isolate FCR3. The complete sequence of the predicted coding region of this protein as well as 3' and 5' non-coding sequences was determined and revealed a 634 amino acid sequence rich in lysine and histidine and containing three distinct, tandemly repeated domains. Indirect immunoflourescence using affinity purified monospecific antibodies directed against recombinant protein expressed in E. coli localizes the KAHRP to the membrane of knobby infected erythrocytes. Immunoelectronmicroscopy established that the protein is clustered on the cytoplasmic side of the erythrocyte membrane and is associated with the electron dense knobs. The role of this protein in mediating cytoadherence was addressed by determining the protein interaction between this protein and other knob associated proteins.

The role of the KAHRP in formation of the knob structure was investigated through the analysis of mutations which lead to the loss of knob formation. K^- mutants arise spontaneously in culture, and can be cloned out of natural infections. The molecular basis for this mutation was investigated in three clonal, K^- isolates. In all cases analyzed, the gene for the KAHRP had undergone a rearrangement, resulting in the deletion of 3' coding sequences. The chromosome to which this gene maps, chromosome 2, had undergone a rearrangement in these mutants, resulting in a telomeric location for the truncated KAHRP. The site of rearrangement in the KAHRP is located at or near the tandemly repeated domain encoding the polyhistidine sequences. Recent studies (to be described below) have focussed on the structure of the recombinant chromosome as well as the structure of both parents in this recombination event. Finally, studies have begun on defining the structure, stability and role of telomeric sequences in generating chromosomal polymorphisms.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

The investigator(s) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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BODY OF REPORT

Three areas of research have been pursued in the last 12 months which summarize the specific goals of this program.

1) Structure of the knob-associated histidine-rich protein (KAHRP) gene in knobby (K+) and knobless (K-) parasites

2) Regulation of expression of the KAHRP in K+ and K- parasites; role of chromosomal location on expression

3) Function of the KAHRP in knob formation and sequestration

BACKGROUND AND SIGNIFICANCE

The virulence associated with P. falciparum malaria results, in part, from the ability of the infected erythrocyte to sequester within the microvasculature. Sequestration favors parasite development by maintaining the organism at low PO_2 (1), and avoiding the filtering action of the spleen (2). This phenotype is partially responsible for the high parasitemias and hypoxia frequently associated with falciparum malaria and may also contribute to the pathophysiology of cerebral malaria (3,4). Sequestration is mediated by electron dense protrusions on the infected erythrocyte surface, referred to as knobs (5), which are the sites of attachment between the infected erythrocyte and the endothelial cells of post capillary venules (6). Spontaneous mutations of knob-expressing parasites have been described which do not cause knob formation and do not cytoadhere (7). Re-introduction of these knobless (K^-) variants in vivo resulted in rapid clearance by the spleen and non-productive infections (8), consistent with a role for the knob in evading splenic defenses. While the knob structures are necessary for cytoadherence, they are not sufficient, since cultured parasites can retain the knob structures but lose the ability to cytoadhere (9). A number of host and parasite proteins are implicated in the cytoadherence phenomenon. Parasite proteins implicated in this phenotype include a knob associated histidine rich protein (KAHRP) which is correlated with the presence of knobs (10), and strain variant, cell-surface, high molecular weight proteins identified by reactivity with immune serum which inhibit cytoadherence (11). Two host proteins have been identified by their ability to mediate the binding of infected erythrocytes to endothelial cells-thrombospondin (12) and an endothelial cell surface protein, gpIV, which is defined by the monoclonal antibody OMK5 (13). Thrombospondin (TSP) is a multifunctional adhesive protein with roles implicated in platelet aggregation (14), cell-to-cell interactions (15) and cell-to-matrix interactions (16,17). TSP binds to a number of macromolecules including fibronectin (18), fibrinogen (19), type V collagen (20), plasminogen (21), heparin (22), the human histidine rich glycoprotein (23) and gpIV (24).

The molecular mechanisms mediating knob formation and cytoadherence have been studied through the detailed analysis of the KAHRP and the regulation if its expression. Structural analysis of this protein his proceeded through the isolation and characterization of the genes which encode this protein. Preliminary protein sequence determination derived from cDNA analysis and overexpression of the KAHRP protein in E. coli has yielded the substrate for

the analysis of the protein interactions which are involved in cytoadherence. Further studies to dissect the role of other host and parasite proteins in knob formation and cytoadherence have followed from those initial observations.

The molecular analysis of several spontaneous K^- mutants revealed that the genetic mechanisms which underly mutation in P. falciparum may be quite unexpected and signficant in parasite survival strategies. Loss of the knob in these K^- mutants results from loss of expression of the KAHRP. A chromosomal rearrangement in these parasites has resulted in the deletion of coding sequences for this gene and relocation of the truncated gene to a telomere. The result of this rearrangement is a hybrid gene consisting of 5' noncoding and coding sequence fused to telomeric sequences. Deletion of coding sequences 3' of the tandemly repeated, polyhistidine encoding sequences has been observed in three independent, clonal K^- isolates. The resulting hybrid gene is transcribed (see below) although mRNA accumulation is not observed, suggesting the presence of an unstable message. Loss of knobs is a lethal phenotype in vivo, resulting in clearance of the infected erythrocytes by the spleen. Thus, the maintenance of this genetic mechanism which gives rise to K^- mutants cannot be selected for this particular phenotype. Rather, it would suggest, that a mechanism which generates chromosomal rearrangement and/or telomeric conversion is of selective advantage in parasite survival in vivo. The selective deletion of sequences or reassortment of chromosomal DNA resulting in altered patterns of expression may be of advantage to the parasite to evade environmental selective pressures. To explore the role of this chromosomal rearrangement to parasite selection, several lines of research are underway. The molecular mechanism of this rearrangement is under investigation through the analysis of both parents of the rearrangement and the resulting recombinant. The generality of such rearrangements is being sought through the characterization of strain-depedent chromosomal polymorphisms, to determine if telomeric rearrangements are involved and if transcribed sequences might be contiguous to such rearrangements. Specific phenotypes like gametocyte production and drug resistance, for which clonal populations of wild type and mutant phenotypes exist will be characterized for the involvement of telomeric rearrangements.

PERSONNEL

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UNPUBLISHED PRELIMINARY STUDIES

A thrombospondin binding domain is expressed in the KAHRP.

In order to begin to define the role of the various host and parasite proteins implicated in the cytoadherence phenotype, interactions between the proteins associated with the knob structure have been mapped. The KAHRP is integral component of this structure and is a likely target for the assembly of other cytoadherence-associated molecules. The binding of TSP to the human histidine-rich glycoprotein (23) suggested the possibility the KAHRP, with a histidine-rich domain, may interact with TSP. To address this possibility, a portion of the KAHRP gene, extending from the BamHI site to the end of cDNA clone LP20 (nucleotide 764-1548, see Fig. 1a & b) containing the 56 amino acid histidine rich region and the adjacent 187 amino acids were overexpressed in E. coli as a fusion protein with NS1, the nonstructural protein of influenza virus,

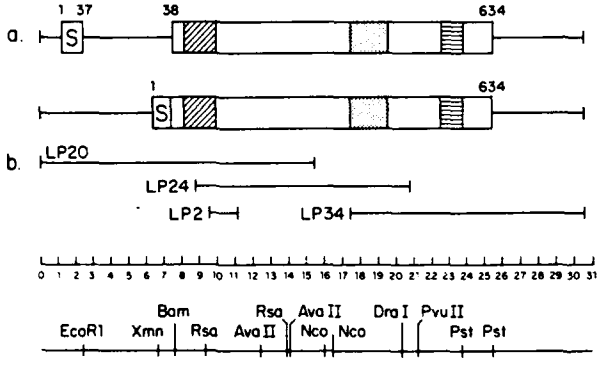

Fig. 1a

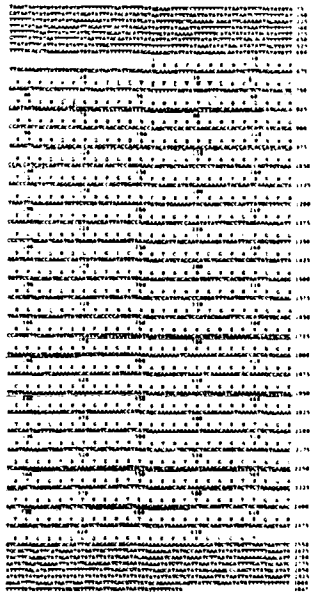

Fig. 1b

yielding a 47,000 dalton protein (Fig. 2). The fusion protein was purified from the bacterial proteins by treatment of the bacteria with 10mM TRIS, pH 8.0, 1% TRITON x-100, followed by sonication. The pellet was washed extensively with the above buffer and then extracted with 5M Urea (Urea fusion lane). The recombinant KAHRP protein thus purified was coupled to an affinity matrix. ^{125}I labeled human TSP and albumin were then incubated with the affinity matrix. After extensive washing, the bound proteins were eluted with high salt concentrations and with low pH. As seen in Fig. 3, TSP binds to the KAHRP and

is eluted in high salt concentrations, while the albumin control does not bind to the affinity column. TSP did not bind to the control protein, NS1, purified from E. coli or to the affinity matrix alone (data not shown).

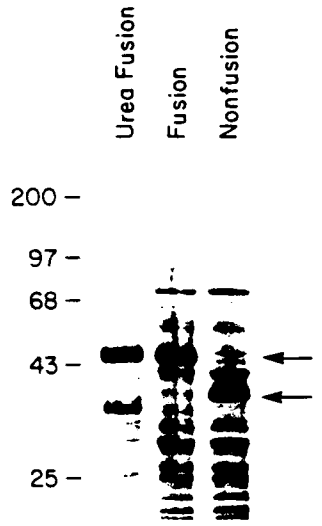

Fig. 2

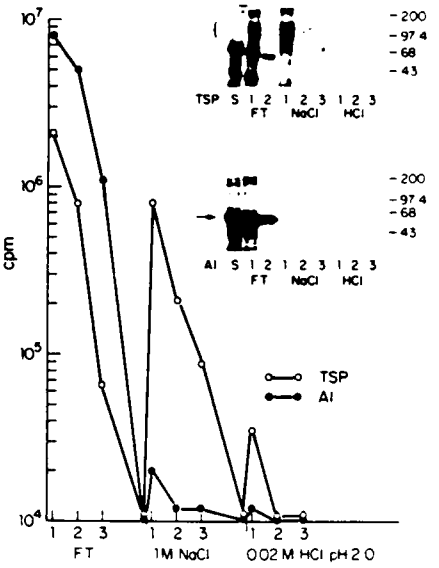

Fig. 3

This binding interaction was demonstrated by an alternative approach, in which the KAHRP protein was immobilized on a plastic surface, then incubated with TSP (19). Saturable TSP binding to KAHRP was detected by ELISA using anti-TSP serum (Fig. 4). A similar result was obtained when TSP was immobilized on plastic (Fig. 5).

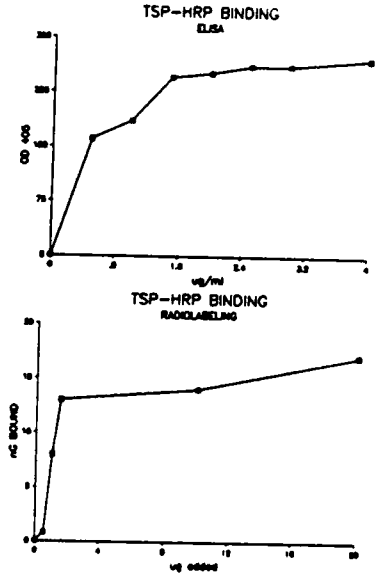

Fig. 4

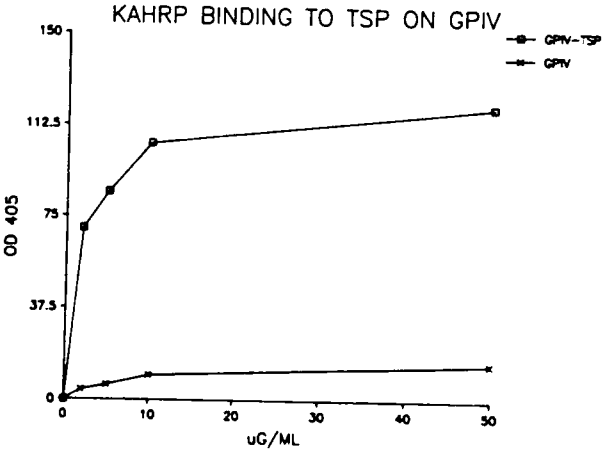

Fig. 5

To further map the domains within the KAHRP responsible for this binding to TSP, sequential deletion mutants were constructed, progressively removing greater amounts of the amino acids carboxy-terminal to the histidine rich region (25). Proteins of 30,000, 28,000, and 21,000 daltons representing constructs of approximately 137, 122, and 71 amino acids of the KAHRP were coupled to an affinity matrix and tested for TSP binding as above. All constructions demonstrated binding to TSP (not shown), establishing that the histidine-rich domain of the KAHRP is capable of binding to TSP.

Chromosomal rearrangement in knobbless parasites

Characterization of the mechanism of chromosome rearrangement in K^- parasites is proceeding through the structural analysis of the parent chromosomes (KAHRP locus on chromosome 2, telomere of chromosome 2) and the resulting recombinant (KAHRP-telomere fusion of knobless parasites). The sequence of the KAHRP locus is shown in Fig. 1b. Cloning of this locus from the K^- mutants was achieved as described (26). Bal 31 digestion renders the telomeric sequence accessible to cloning, facilitating their introduction into standard vectors. Multiple independent clones were isolated with this strategy and characterized. The partial sequence of one such clone is shown in figure 6. The rearrangement break point occurs 30 bp 5' of the tandemly repeated polyhistidine-encoding sequence. Three repeat elements have been identified in the telomeric sequences. Region I shows strong homology to the consensus telomere sequence found in yeast and is very similar to a telomere sequence reported for P. berghei. This telomere sequence is conserved in all P. falciparum chromosomes identified by PFG analysis, as shown in figure 7, in which all separated chromosomes hybridize with the probe derived from the telomere of figure 6. Characterization of the chromosome 2 telomere is proceeding according to the strategy outlined above. DNA has been isolated from chromosome 2 separated by PFG, digested with Bal 31 and cloned into appropriate vectors. Sequence analysis is currently in progress. These chromosome 2 telomere clones will be used to distinguish among the various models which can account for this rearrangement.

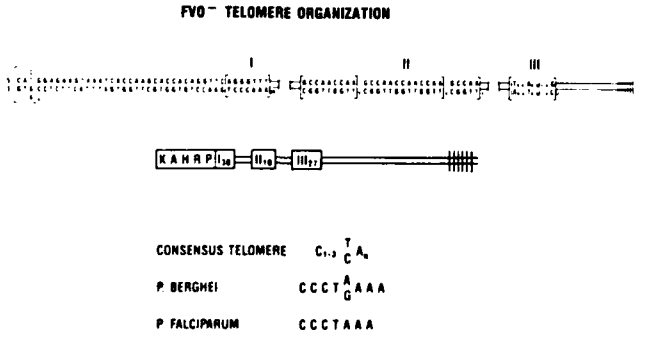

Fig. 6

Fig. 7

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25. The expression vector $pB4^+$ containing a 784 nucleotide portion of the KAHRP gene (40) was linearized with Bgl II which cuts 3' of the insert, then treated with the exonuclease Bal 31 (NEB) (0.75 units Bal 31/15µg DNA) for minute intervals. The reaction was stopped with 20mM EGTA. The 3' recessed termini were filled in by the Klenow fragment of DNA polymerase I (NEB), then ligated and transformed into E. coli AR58 and induced as described (21). Induced extracts were fractionated on 12% SDS-PAGE and the resulting truncated fusion proteins identified.

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